Ki67 Antigen Expression Correlates with Tumor Progression and HLA-DR Antigen Expression in Melanocytic Lesions

Silvia Moretti, Roberta Massobrio, Laura Brogelli, Mauro Novelli, Benvenuto Giannotti, and Maria Grazia Bernengo

Clinica Dermatologica II (SM, LB, BG), University of Florence, Florence, Italy and Clinica Dermatologica I (RM, MN, MGB), University of Turin, Turin, Italy

Advanced steps of tumor progression are generally characterized by an increased growth fraction within the neoplastic cell population. The presence of a relevant growth fraction is also related to widely accepted prognostic parameters in some human malignancies. Our aims were to evaluate the presence of a growth fraction with Ki67 monoclonal antibody (MoAb), and to correlate it with tumor progression and HLA-DR antigen expression in 88 melanocytic lesions. The lesions were 19 acquired melanocytic nevi, 58 primary melanomas [divided into 26 superficial spreading melanomas (SSM), 24 superficial spreading melanomas with nodular areas (SS + NM), and five nodular melanomas (NM)], and 11 metastases from malignant melanomas. Ki67 MoAb stained 16%, 19%, 71%, 100%, and 82% of nevi, SSM, SS + NM, NM, and metastases, respectively. Among primary melanomas, Ki67 MoAb stained 12%, 28%, 50%, and 70% of tumors <0.75, 0.75–1.49, 1.5–2.9, and ≥ 3 mm thick, respectively. A concordant reactivity pattern for Ki67 and HLA-DR antigens was found in 72% of lesions ($p < 0.0001$). We have shown that a representative growth fraction (ie, Ki67 reactivity) is present in melanocytic lesions only in advanced steps of tumor progression and correlates with HLA-DR antigen expression. Despite the different biologic values of Ki67 and HLA-DR antigens, we suggest the joint evaluation of both antigens as a useful marker of aggressive behavior in melanoma. *J Invest Dermatol 95:320–324, 1990*

Tumor progression is a multistep process leading to clinically relevant events such as local invasion and metastases. Such a phenomenon is generally characterized by the appearance within the tumor of new-cell subpopulations expressing some selective growth advantage [1,2]. In fact, advanced tumor cell populations tend to increase their growth rate and escape from local growth-control mechanisms. Interestingly, the increase in the growth rate seems not to reflect a shortening of the cell cycle time, but rather an increase in the growth fraction that is the proportion of neoplastic cells that continue to proliferate actively instead of differentiating or progressing to cell death [1]. Therefore, the evaluation of the growth fraction may be useful in understanding the evolutionary phase of a single tumor. Moreover, the growth fraction seems closely related to already known prognostic parameters and to the course of the disease in some malignancies, including breast cancer, acute leukemias, and lymphomas [3–7].

Among the immunologic markers of proliferation rate, Ki67 monoclonal antibody (MoAb), recognizing a nuclear antigen expressed in proliferating cells, is considered a reliable tool in evaluating the growth fraction [8–10]. Ki67 MoAb can be applied to frozen sections and, with the use of currently employed immunohistochemical techniques, to serial sections, where both the growth fraction and the expression of some antigenic markers can be evaluated.

We assessed the growth fraction using Ki67 MoAb in a group of melanocytic lesions in different evolutionary phases, including melanocytic nevi, primary melanomas, and metastatic melanomas. Our aims were to evaluate the growth fraction in different steps of progression of melanocytic lesions and to correlate the presence of a representative growth fraction with tumor progression. In addition, because HLA-DR antigen expression has been demonstrated to correlate with advanced stages of tumor progression in melanoma [2,11–15], we evaluated the correlation of the growth fraction with the expression of HLA-DR antigens.

MATERIALS AND METHODS

Melanocytic Lesions Eighty-eight melanocytic lesions from 86 patients were examined. Among the lesions were 19 melanocytic acquired nevi (18 common acquired nevi and one Spitz nevus), 58 primary melanomas, and 11 melanoma metastases (five nodal and six cutaneous). Primary melanomas were divided into 3 groups according to clinical and histologic evaluations [16]. In the first group, there were 26 melanomas appearing clinically as plaques and show-

Manuscript received April 19, 1989; accepted for publication January 9, 1990.

Dr. L. Brogelli was supported by a contribution of the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Milan, Italy.

This work was presented in part at the 18th Annual Meeting of the ESDR, Munchen, 1988.

Reprint requests to: Dr. Silvia Moretti, Clinica Dermatologica II, via della Pergola 58, 50121, Firenze, Italy.

Abbreviations:
MET: metastatic melanoma
MoAb: monoclonal antibody
NCM: non-classified melanoma
NM: nodular melanoma
SS + NM: superficial spreading melanoma with a nodular area
SSM: superficial spreading melanoma

0022-202X/90/$03.50

Table I. Case Definition and Immunostaining Results

Acquired Melanocytic Nevi

Case Number	Histologic Type	% Cells Ki67 +	% Cells HLA-DR +
1	Compound	0	0
2	Compound	0	0
3	Compound	0	0
4	Compound	0	0
5	Compound	0	0
6	Compound	5	0
7	Intradermal	0	0
8	Compound	0	0
9	Intradermal	0	0
10	Intradermal	0	0
11	Junctional	0	0
12	Intradermal	0	0
13	Intradermal	0	0
14	Compound	0	0
15	Compound	0	0
16	Compound	0	0
17	Compound	0	0
18	Junctional	0	0
19	Spitz	8	0–10+[a]

Primary Melanomas

Case Number	Tumor Thickness	Histotype	% Cells Ki67 +	% Cells HLA-DR +
1	0.25	SSM	0	11–30
2	0.30	SSM	30	11–30
3	0.43	SSM	2	0–10+
4	0.50	SSM	0	0–10
5	0.55	SSM	2	0–10
6	0.60	SSM	2	0–10
7	0.60	SSM	0	0–10
8	0.70	SSM	0	0–10
9	0.80	SSM	2	0–10
10	0.95	SSM	1	0–10
11	1.08	SSM	0	0–10
12	1.10	SSM	2	0–10
13	1.20	SS + NM	11	0–10
14	1.20	SSM	0	0–10
15	1.30	SSM	0	0–10
16	1.40	SS + NM	30	0–10
17	1.50	SS + NM	1	0–10+
18	1.50	SS + NM	11	11–30
19	1.65	SSM	2	0–10
20	1.75	SSM	0	0–10
21	1.80	SSM	0	0–10
22	1.90	SS + NM	8	11–30
23	2.00	SS + NM	11	11–30
24	2.08	SS + NM	1	0–10
25	2.08	SSM	5	0–10

Primary Melanomas

Case Number	Tumor Thickness	Histotype	% Cells Ki67 +	% Cells HLA-DR +
26	2.26	SSM	1	0–10+
27	2.40	SS + NM	50	0–10
28	2.50	SS + NM	0	0–10
29	2.55	SSM	5	11–30
30	2.70	SS + NM	3	0–10
31	3.00	SS + NM	10	0–10+
32	3.10	SS + NM	2	11–30
33	3.20	SS + NM	3	0–10
34	3.50	SSM	10	31–50
35	3.50	NM	5	31–50
36	3.50	NCM	0	0–10
37	3.50	SS + NM	0	0–10
38	3.90	NCM	11	0–10
39	3.96	SSM	1	0–10
40	4.00	SS + NM	50	0–10
41	4.00	SS + NM	20	11–30
42	4.05	SS + NM	5	0–10+
43	4.20	SSM	0	0–10
44	4.25	SS + NM	0	0–10
45	4.30	SS + NM	40	31–50
46	4.30	SS + NM	1	0–10+
47	4.40	NCM	3	0–10
48	4.75	SSM	5	0–10+
49	5.20	SS + NM	70	0–10+
50	5.60	SS + NM	8	11–30
51	5.90	SS + NM	30	0–10
52	5.90	NM	10	0–10
53	6.00	NM	20	0–10+
54	6.00	SSM	2	0–10+
55	7.20	SS + NM	11	11–30
56	7.35	NM	10	0–10
57	15	NM	15	11–30
58	Not evaluable	SSM	0	31–50

Metastases

Case Number	Site	% Cells Ki67 +	% Cells HLA-DR +
1	Skin	10	31–50
2	Skin	0	0–10
3	Skin	10	11–30
4	Skin	0	0–10
5	Skin	15	11–30
6	Skin	50	0–10
7	Nodal	3	31–50
8	Nodal	30	31–50
9	Nodal	25	0–10
10	Nodal	5	0–10
11	Nodal	10	0–10

[a] +, Clusters of stained tumor cells.

ing radial growth phase patterns on microscopic examination [superficial spreading melanoma (SSM)]. In the second group, there were 24 melanomas appearing clinically as plaques plus nodule(s), and showing both radial growth-phase patterns and vertical growth-phase patterns [superficial spreading melanoma with a nodular area (SS + NM)] on microscopic examination. In the third group, there were five melanomas appearing as nodules and with vertical growth-phase patterns on microscopic examination [nodular melanomas (NM)].

Three additional melanomas appeared clinically as plaques plus a nodule but could not be evaluated according to histologic type. These three cases were, however, evaluated when tumor thickness was assessed. Primary melanomas were divided into four groups according to Breslow [17]: eight tumors were less than 0.75 mm thick, 8 were 0.75–1.49 mm thick, 14 were 1.5–2.9 mm thick, and 27 were ≥3 mm thick. The thickness of one SSM could not be evaluated, and therefore this case was included only in the evaluation by histologic type. Case definition is reported in Table I together with immunostaining results.

MoAb and Staining Assay The proliferating cells were stained with Ki67 MoAb, which reacts with a nuclear antigen expressed by cycling cells (Dakopatts, Copenhagen, Denmark) [8]. HLA-DR antigens were identified with an anti–HLA-DR MoAb purchased from Becton Dickinson (Mountain View, CA, USA). An indirect immunoperoxidase assay was used. Acetone-fixed cryostat sections were incubated with MoAb for 2 h (Ki67 MoAb) or 1 h (anti–HLA-DR MoAb); they were later incubated with goat biotinylated Fab-antimouse serum (Amersham, Buckingamshire, UK) and then with streptavidin-biotin-peroxidase complex (Amersham). The peroxidase reaction was performed with 3-amino-9-ethyl carbazole and H_2O_2 in acetate buffer. Counterstaining was done with Mayer's hematoxylin. Negative controls were run in parallel. The positive

reaction of Ki67 MoAb with normal epidermis in the same section of each lesion served as an internal control.

Evaluation The percentage of stained cells in each section was evaluated by two independent observers. In most cases, the two observers agreed; when differences were present, they were resolved by consensus. The growth fraction was assessed as the percentage of cells reacting with Ki67 MoAb in the whole section. Sections were indicated as positive when at least 3% of tumor cells were stained with Ki67 MoAb (such lesions were considered to have a representative growth fraction), and when at least 11% or clusters of tumor cells were stained with anti-HLA-DR MoAb. HLA-DR+ melanocytic cells were carefully differentiated from HLA-DR+ cells belonging to the reactive infiltrate with use of OKT11 (Ortho Diagnostic System, Raritan, NJ) and anti-LCA (Dako) MoAb on serial sections.

Statistical Analysis The χ^2 test was used.

RESULTS

The histopathologic features and results of immunostaining for Ki67 and HLA-DR antigens of 88 melanocytic lesions are listed in Table I, and representative staining in two cases is illustrated in Figs 1 and 2.

Figures 3 and 4 show the percentage of Ki67-stained cells per lesion. Melanocytic lesions are divided according to histopathologic features (Fig 3) and primary melanomas according to tumor thickness (Fig 4). When a vertical growth pattern is present in primary melanomas, higher growth fractions are seen (Fig 3); similar values are observed among metastases. Although the highest values of growth fraction are observed in thick primary melanomas, a correlation between growth fraction and tumor thickness was not found (Fig 4). Figures 5 and 6 show the number of Ki67 and HLA-DR–positive lesions classified according to the histopathologic features of melanocytic lesions or to tumor thickness of primary melanomas, respectively. Figure 5 shows that 16%, 19%, 71%, 100%, and 82% of nevi, SSM, SS + NM, NM, and metastases, respectively, were positively stained by Ki67 MoAb ($\chi^2 = 34.8$; $p < 0.000001$). Figure 6 shows that 12%, 28%, 50%, and 70% of primary melanomas <0.75, 0.75–1.49, 1.5–2.9, and ≥3 mm thick, respectively, reacted positively with Ki67 MoAb. Due to the fact that the χ^2 test was not applicable because the numbers were too small, we tested melanomas less than 3 mm thick vs melanomas more than 3 mm

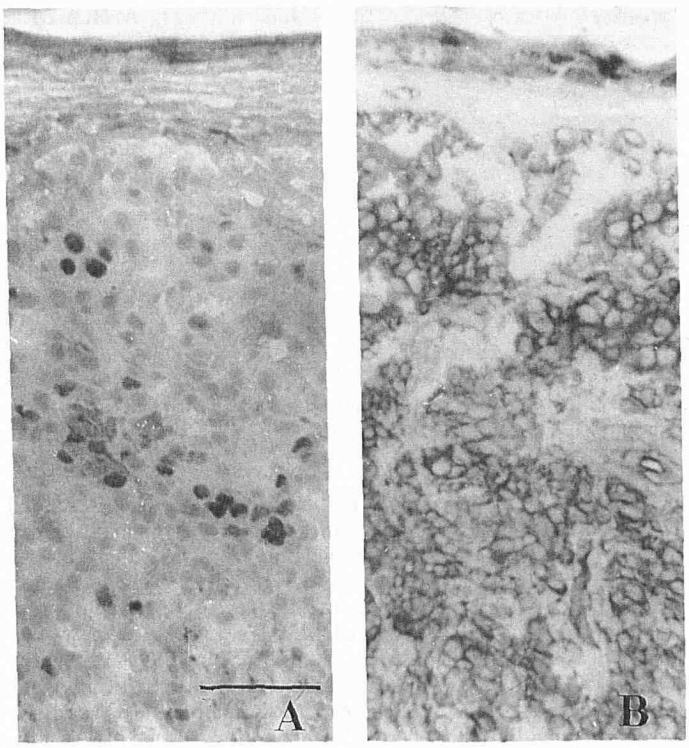

Figure 1. A superficial spreading melanoma with a nodular area. *A*, staining of melanoma cells with Ki67 MoAb; *B*, staining of melanoma cells in the same area with anti-HLA-DR MoAb. *Bar*, 100 μm.

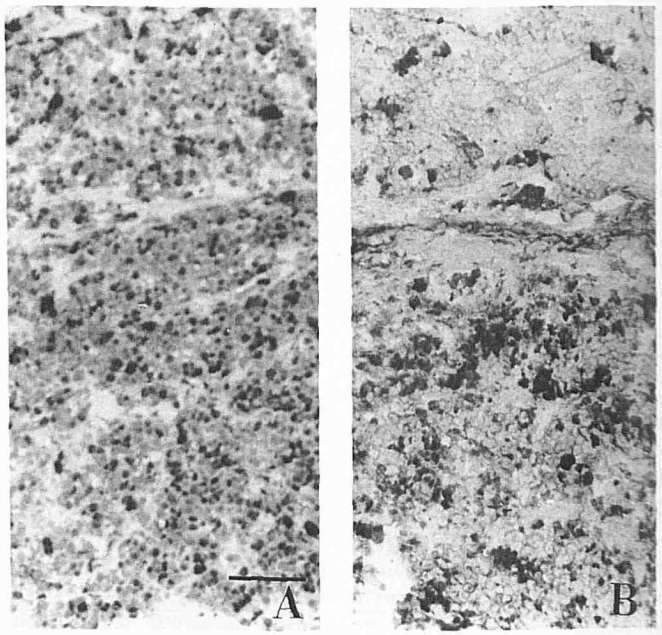

Figure 2. A cutaneous melanoma metastasis. *A*, melanoma cells stained with Ki67 MoAb; *B*, reactive infiltrating cells are stained with anti HLA-DR MoAb, whereas melanoma cells are not stained. *Bar*, 100 μm.

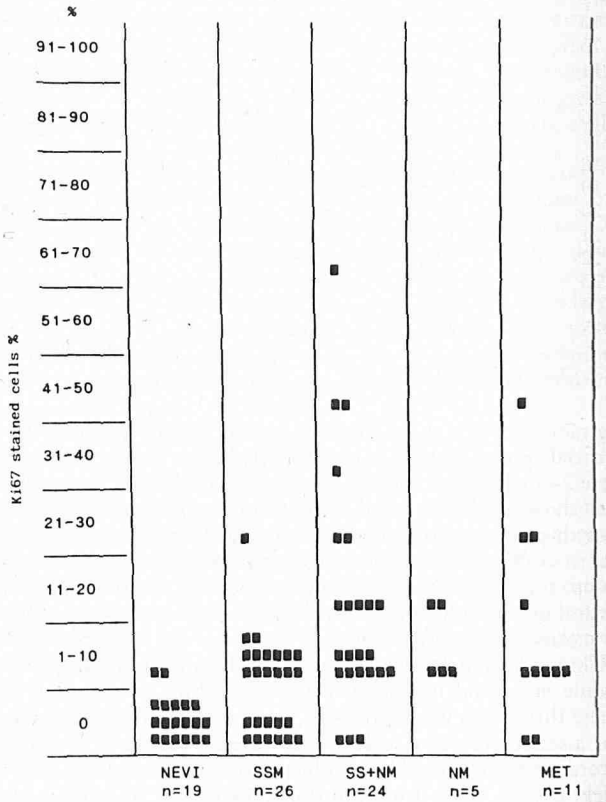

Figure 3. Growth fraction in melanocytic lesions expressed as percentage of Ki67-stained cells per each lesion.

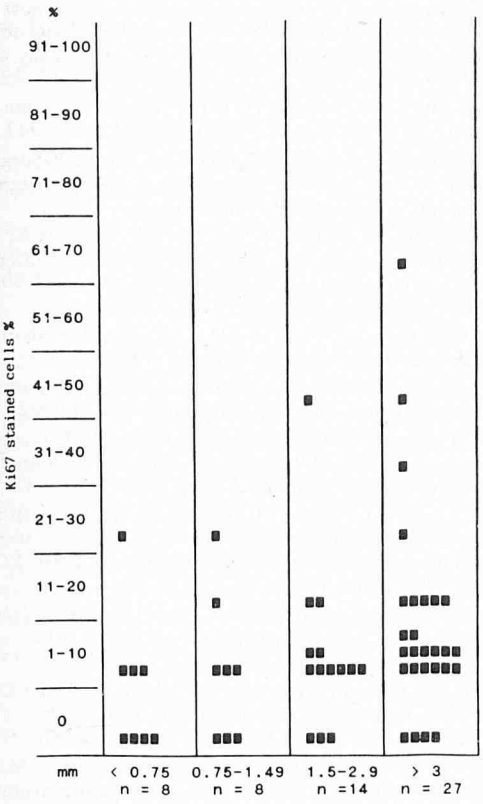

Figure 4. Growth fraction in primary melanomas expressed as percentage of Ki67 stained cells per each lesion.

thick only. ($\chi^2 = 7.8$; $p = 0.0049$.) HLA-DR reactivity was found in 5%, 35%, 54%, 60%, and 45% of nevi, SSM, SS + NM, NM, and metastases, respectively ($\chi^2 = 7.64$; $p = 0.007$) (Fig 5), and in 37%, 0%, 47%, and 56% of primary melanomas <0.75, 0.75–1.49, 1.5–2.9, >3 mm thick, respectively ($\chi^2 = 8.98$; $p = 0.029$) (Fig 6). A concordant reactivity pattern for Ki67 and HLA-DR antigens was found in 72% of lesions. Both antigens were expressed in 26% of lesions and neither of them was present in 45% of lesions (Table II). The degree of concordance was statistically significant ($p < 0.0001$).

DISCUSSION

We have demonstrated that the increased frequency of a representative growth fraction identified by Ki67 MoAb was associated with tumor progression in melanocytic lesions. In fact, a representative growth fraction was observed among 19 nevi in only two lesions, one of them a Spitz nevus. Among primary melanomas, positive

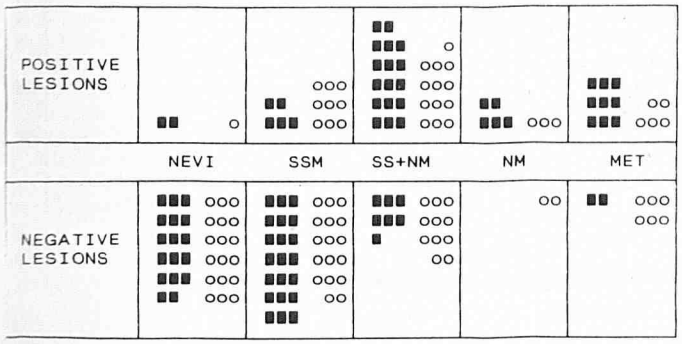

Figure 5. Positive and negative staining of melanocytic lesions for Ki67- *(solid squares)* and HLA-DR- *(open circles)* antigen expression.

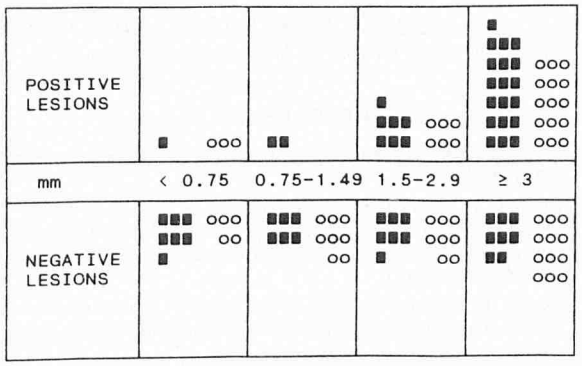

Figure 6. Positive and negative staining of primary melanomas for Ki67- *(solid squares)* and HLA-DR- *(open circles)* antigen expression.

Ki67 staining was seen with increasing frequency according to increasing tumor thickness and according to histotype. Among 11 metastases, positive staining was found in all but two lesions. In addition, we observed a correlation between Ki67 antigen expression and HLA-DR antigen expression, which is considered a progression marker [2,11–15]. Ki67 score was found to be an objective indicator of biologic behavior in breast cancer [5,18–20] and to be of some prognostic significance in non-Hodgkin's lymphoma [7,9]. In melanocytic lesions, Ki67 staining has been correlated with the malignant potential of congenital nevi [21] and with histopathologically assessed prognostic variables in melanoma [22,23]. Previous studies of tumor cell kinetics, performed mainly on metastatic melanoma, actually showed that stage II patients with tumors with a high labeling index had a lower probability for two years of survival [24]. Moreover, the S-fraction determined by flow cytometry was proved to be an important indicator of overall survival [25]. At present, we are not able to assess the value of Ki67 score as a prognostic factor in melanoma because the follow-up period of our patients has not been long enough. In our study, we found no significant association between Ki67 antigen expression and clinical parameters such as age, sex of patients, and site of the primary melanoma, but we found a clear correlation of Ki67 antigen expression and tumor thickness, which is a proved indicator of a poor prognosis. It was, however, recently pointed out, in a large series of patients, that tumor thickness is not a reliable prognostic factor for thin primary melanoma (ie, thinner than 1.5 mm) [26]. It would be interesting to establish, by means of an adequate follow-up, whether Ki67 score is of prognostic significance for thin melanomas.

At present, 12 of 58 stage I patients have had a relapse; 6 of 12 had both Ki67 and HLA-DR antigens expressed in the primary tumor, nine of 12 expressed Ki-67 antigen, and three of 12 expressed HLA-DR antigen. Thus 11 of 12 expressed at least one of the two antigens. In addition, four of five patients who have died had a primary melanoma with a high percentage of Ki-67–stained tumor cells. It is noteworthy that one of these patients had the only thin primary melanoma of the present series (0.3 mm, II level, with regression), with a high percentage of Ki-67–stained tumor cells (30%). Therefore, despite the different biologic values of Ki67 and HLA-DR antigens, we suggest the joint evaluation of both antigens as a useful marker of aggressive behavior in melanoma.

Table II. Concordance of Ki67 and HLA-DR Antigen Expression in 88 Melanocytic Lesions

	Ki67 +	Ki67 −	
HLA-DR +	23	8	31
HLA-DR −	17	40	57
Total	40	48	88

We wish to express our gratitude to Prof. P. Urbano, chief of the Institute of Microbiology, University of Florence, for his statistical suggestions.

REFERENCES

1. Nowell PC: Mechanisms of tumor progression. Cancer Res 46:2203–2207, 1986
2. Holtzman B, Bröcker EB, Lehmann JM, Ruiter DJ, Sorg C, Riethmuller G, Johnson J: Tumor progression in human malignant melanoma: five stages defined by their antigenic phenotypes. Int J Cancer 39:466–471, 1987
3. Costa A, Bonadonna G, Villa E, Valagussa P, Silvestrini R: Labeling index as a prognostic marker in non-Hodgkin's lymphoma. J Natl Cancer Inst 66:1–5, 1981
4. Smets LA, Taminian J, Hahlen K, de Waal F, Behrendt H: Cell kinetic responses in childhood acute non lymphocytic leukemia during high-dose therapy with cytosine arabinoside. Blood 61:79–84, 1983
5. Barnard NJ, Hall PA, Lemoine NR, Kedar N: Proliferative index in breast carcinoma determined in situ by Ki-67 immunostaining and its relationship to clinical and pathological variables. J Pathol 152:287–295, 1987
6. Shepherd NA, Richman PI, England J: Ki-67 derived proliferative activity in colorectal adenocarcinoma with prognostic correlations. J Pathol 155:213–219, 1988
7. Hall PA, Richards MA, Gregory WM, d'Ardenne AJ, Lister TA, Stansfeld AG: The prognostic value of Ki-67 immunostaining in non-Hodgkin's lymphoma. J Pathol 154:223–235, 1988
8. Gerdes J, Schwab U, Lemke H, Stein H: Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–20, 1983
9. Gerdes J, Dallenbach F, Lennert K, Lemke H, Stein H: Growth fractions in malignant non-Hodgkin's lymphomas as determined in situ with the monoclonal antibody Ki-67. Haematol Oncol 2:365–371, 1984
10. Pileri S, Gerdes J, Rivano M, Tazzari PL, Magnani M, Gobbi M, Stein H: Immunohistochemical determination of growth fractions in human permanent cell lines and lymphoid tumors: a critical comparison of the monoclonal antibodies OKT9 and Ki-67. Br J Haematol 65:271–276, 1987
11. Ruiter DJ, Bhan AK, Harrist TJ, Sober AJ, Mihm MC: Major histocompatibility antigens and mononuclear inflammatory infiltrate in benign nevomelanocytic proliferations and malignant melanoma. J Immunol 129:2808–2815, 1982
12. Ruiter DJ, Bergman W, Welvaart K, Scheffer E, van Vloten WA, Russo C, Ferrone S: Immunohistochemical analysis of malignant melanomas and nevocellular nevi with monoclonal antibodies to distinct monomorphic determinants of HLA antigens. Cancer Res 44:3930–3935, 1984
13. Bröcker EB, Suter L, Sorg C: HLA-DR antigen expression in primary melanomas of the skin. J Invest Dermatol 82:244–247, 1984
14. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C: Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36:29–33, 1985
15. van Vreeswijk H, Ruiter DJ, Bröcker EB, Welvaart K, Ferrone S: Differential expression of HLA-DR, DQ and DP antigens in primary and metastatic melanoma. J Invest Dermatol 90:755–760, 1988
16. Clark WH, Elder DE, Van Horn M: The biologic forms of malignant melanoma. Hum Pathol 17:443–450, 1986
17. Breslow A: Thickness cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg 172:902–908, 1970
18. Lellé RJ, Heidenreich W, Steuch G, Gerdes J: The correlation of growth fractions with histologic grading and lymphnode status in human mammary carcinoma. Cancer 59:83–88, 1987
19. Gerdes J, Lellé RJ, Pickartz H, Heidenreich W, Schwarting R, Kurtsiefer L, Stanch G, Stein H: Growth fractions in breast cancers determined in situ with monoclonal antibody Ki-67. J Clin Pathol 39:977–980, 1986
20. Lellé RJ, Heidenreich W, Stanch G, Wecke I, Gerdes J: Determination of growth fractions in benign breast disease (BBD) with monoclonal antibody Ki-67. J Cancer Res Clin Oncol 113:73–77, 1987
21. Steijlen PM, Hamm H, van Erp PEJ, Johson JP, Ruiter DJ, Brocker EB: Immunohistologic evidence for the malignant potential of congenital melanocytic nevi. J Invest Dermatol 92:366–370, 1989
22. Kaudewitz P, Braun-Falco O, Ernst M, Landthaler M, Stolz W, Gerdes J: Tumor cell growth fractions in human malignant melanomas and the correlation to histopathologic tumor grading. Am J Pathol 134:1063–1068, 1989
23. Smolle J, Soyer HP, Kerl H: Proliferative activity of cutaneous melanocytic tumors defined by Ki67 monoclonal antibody. Am J Dermatopathol 11:301–307, 1989
24. Costa A, Silvestrini R, Grignolio E, Clemente C, Attili A, Testori A: Cell kinetics as a prognostic tool in patients with metastatic malignant melanoma of the skin. Cancer 60:2797–2800, 1987
25. Hanson J, Tribukait B, Lewensohn R, Ringborg U: Flow cytofluorometric DNA analysis of metastases of human malignant melanomas. Anal Quant Cytol 4:99–104, 1982
26. Knuehln-Petzoldt C, Fisher S: Tumor thickness is not a prognostic factor in thin melanoma. Arch Dermatol Res 279:487–488, 1987